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Characterization of Skin Symbiotic Bacteria of Sympatric Amphibians in Southeastern China

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Abstract The fungal infection called chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*), has given rise to dramatic declines or extinctions of many amphibian species around the world; however, in Asia, this disease has shown a low zoospore load or scant mortality. One potential reason for this may be that certain unique community structures of amphibian skin symbiosis contribute to the outcome of the disease; nevertheless, we know very little about the microbiota in this region. In this study, we used skin swabs of five sympatric amphibian species that have various habitat preferences in Lishui, Zhejiang Province, a place in southeastern China, to explore the skin bacterial communities by using 16S rRNA amplicon sequencing. We detected a total of 1020 OTUs, belonging to 17 phyla, among which Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes dominated all five host species. Enterobacteriaceae and Exiguobacteraceae and the genera *Escherichia* and *Exiguobacterium* belonging to these two families were identified as the most abundant taxa on our focal species. The alpha diversity was significantly lower on the terrestrial species, and also the highly enriched Proteobacteria was found on the terrestrial species, *Rana zhenhaiensis*, whereas Actinobacteria, Bacteroidetes, Cyanobacteria and Firmicutes were more abundant on aquatic species than on the terrestrial species. Our results suggest that both host species and habitat sites are important factors driving skin microbial diversity and composition and that amphibians in China may harbour unique skin bacterial communities. This study helps elucidate amphibian skin microbial ecology, and with further efforts, the specific mechanism of the interaction between *Bd* and host amphibians in China could be elucidated.

Keywords bacterial communities, ecomorphology, frog, microbiome, skin

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1. Introduction

Vertebrates act as habitats for diverse and complex microbial communities that can contribute to host health or, conversely, be detrimental to the host. This holobiont (host plus symbionts) has aroused widespread concern for the past few years (Rosenberg and Zilber-Rosenberg, 2016), such as in studies on different parts of the human body, for example, the intestine (Brooks *et al*., 2018; Vitetta *et al*., 2018), mouth (Vogtmann *et al*., 2019), and vagina (Bernabeu *et al*., 2019; Fettweis *et al*., 2019). In addition, the skin is the largest organ of the body, along with microbiome on it, is a complex and dynamic ecosystem and is given great importance in human studies (Chen *et al*., 2018; Byrd *et al*., 2018). In recent years, the development of next generation sequencing technology allowing such studies about skin microbial symbionts has also been performed in animals, such as mammals (Hoffmann *et al*., 2014; Older *et al*., 2017), birds (Roggenbuck *et al*., 2014; Engel *et al*., 2018), reptiles (Hyde *et al*., 2016; Allender *et al*., 2018; Walker *et al*., 2019), fish (Carda-Diéguez *et al*., 2017; Minniti *et al*., 2017) and, especially, amphibians (McKenzie *et al*., 2012; Kueneman *et al*., 2014; Jani *et al*., 2017; Medina *et al*., 2019). Amphibian skin is among the best-studied systems due to the notorious fungal infection known as chytridiomycosis caused by *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Berger *et al*., 1998; Longcore *et al*., 1999; O'Hanlon *et al*., 2018).

Batrachochytrium dendrobatidis has been linked to dramatic declines of more than 500 amphibian species around the world, amounting for 6.2% of the 8118 described amphibian species, 90 of which have been extinction (Scheele *et al*., 2019; AmphibiaWeb, 2020). An increasing number of studies have found that the skin microbiome of amphibians provides the first line of defence against *Bd* through the production of anti-fungal compounds such as violacein, indole-3-carboxaldehyde, 2,4- diacetylphloroglucinol (DAPG) and prodigiosin (Brucker *et al*., 2008a, b; Harris *et al*., 2009; Woodhams *et al*., 2018; Madison *et al*., 2019). Previous studies on amphibian skin symbiosis

have mainly focused on Australia, Mesoamerica and America, where most amphibian decline events have occurred, while this phenomenon has been less frequently reported in Asia (Bataille *et al*., 2018; Scheele *et al*., 2019). Despite the widespread occurrence of *Bd* in Asia (Goka *et al*., 2009; Swei *et al*., 2011; Bai *et al*., 2012; Zhu *et al*., 2014, 2016), there remains a lack of observation of large amounts of individual deaths or amphibian population declines (Olson *et al*., 2013; Scheele *et al*., 2019). One potential explanation is that there may be certain unique community structures involved in amphibian skin symbiosis that contribute to low *Bd* load or decreased mortality. Therefore, exploring the characterization and investigation of the environmental correlations of the skin microbiota symbionts of native amphibians in Asian regions is crucial for understanding the natural resistance of these animals to *Bd*.

There are still limited number reports on the skin microbiota symbionts of amphibians in Asia. In mainland China, previous studies have mainly focused on *Rana dybowskii* in northeastern China; this is an economically important frog species because its oviduct can be used in traditional Chinese medicine. These studies discussed the differences in skin symbiotic bacteria between farmed and wild individuals and seasonal changes in the gut and skin microbiotas of *R. dybowskii* (Bie *et al*., 2019; Tong *et al*., 2019). Zhao *et al*. (2019) cloned the 16S rRNA gene to conduct bacterial community analysis of the skin of *Odorrana grahami*, a frog mainly distributed in the Yun-Gui Plateau. Studies on skin symbionts of frogs in other parts of China are still scarce, and most studies have focused on only one species. Previous research has shown that amphibian taxonomy (host species) is an important predictor of the diversity and community composition of skin bacteria (McKenzie *et al*., 2012; Kueneman *et al*., 2014; Bletz *et al*., 2017a), and geography (geographical regions), ontogeny (life histories) and ecomorphology (habitats) are also important predictors of the skin microbiome (Abarca *et al*., 2018; Longo *et al*., 2015; Bletz *et al*., 2017b).

Here, we examined the skin bacteria of five sympatric amphibian species from Lishui, Zhejiang Province, a place with no alien amphibian invasions (Liu and Li, 2009; Liu *et al*., 2013) and a high diversity of amphibian species endemic to southeastern China. These five species have various habitat preferences, with three species, namely, *Fejervarya multistriata*, *Hylarana guentheri* and *Microhyla fissipes*, belonging to the aquatic type, generally inhabiting standing water, and one species, *Polypedates megacephalus*, belonging to the arboreal type, often living in low shrubs, grasses or agricultural crops and approaching standing waters during breeding seasons from April to June. The final species, *Rana zhenhaiensis*, is a terrestrial frog endemic to China, with adult animals inhabiting forest grasslands and using standing waters for reproduction only in mating seasons from approximately January to April. Their diverse habitat use provides us with an ideal opportunity to investigate and compare the diversity of the skin bacterial communities of sympatric amphibian species, which will be helpful for future research on the relationship of the skin microbiome with *Bd* prevalence in southeastern China.

2. Materials and Methods

2.1. Sample collection Our study was conducted in April 2019, and we randomly sampled individuals of each of five species (*F. multistriata, H. guentheri, M. fissipes, P. megacephalus, R. zhenhaiensis*) from Lishui, Zhejiang Province. The five species are mainly distributed in eastern and southern China and some countries in southeastern Asia. They showed low or no *Bd* prevalence and no evidence of *Bd*-induced mortality events in both historical specimens from museum and specimens from the wild (Bai *et al*., 2012; Zhu *et al*., 2014) (for sample information, see Table 1). Similar to *F. multistriata*, *H. guentheri* and *M. fissipes*, *P. megacephalus* was also sampled near water because the sampling time was its spawning season, while *R. zhenhaiensis* was sampled on a hillside, away from the water. We followed standard procedures and captured each individual by hand using new disposable polyethylene gloves and then poured ~100 mL of sterile purified water to remove dirt and transient bacteria from the surface of each frog (Belden *et al*., 2015). After flushing, we swabbed each frog with a total of 30 strokes, including five strokes on each side of the abdominal midline, the inner thighs and the foot webbings of each hind leg (Vredenburg *et al*., 2010). Swabs were stored individually in labelled sterile Eppendorf tubes in a car refrigerator before they were stored at –20 °C in the laboratory for further processing.

2.2. Bacterial DNA extraction and sequencing Bacterial genomic DNA was extracted from swabs using the E.Z.N.A. Bacterial DNA Kit (catalogue No. D3350-02; Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol with minor adjustments in the pretreatment of the samples. We first immersed the swabs in 1mL of 1× TE buffer, vortexed and discarded the swabs, centrifuged the suspensions at 10000×g for 3 min, and discarded the supernatant. Then, we added 100 µl of 1× TE buffer, vortexed the mixture to completely resuspend the pellet and followed the protocol provided by the kit. The extracted DNA was quantified by a NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted to 5 ng/µl using RNase-free water. Then, the hypervariable V4 region of the 16S rRNA gene of symbiotic bacteria was amplified using the barcoded primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (barcode-515F and barcode-806R) (Knutie *et al*., 2017). The final amplicon reaction contained 5× FastPfu Buffer (100 mM Tris-SO_4, 200

mM KCl, 50 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 10% glycerol) (TransGen, Beijing, China), 2.5 mM dNTPs, 2.5 U/μl FastPfu DNA Polymerase (TransGen, Beijing, China), 20 ng of DNA, 10 μM barcode primers and PCR grade water to 50 μl. The PCRs included a denaturing step at 95℃ for 2 min; 35 cycles of 95 ℃ for 20 s, 51 ℃ for 20 s, and 72 ℃ for 30 s; and a final extension for 5 min at 72 ℃. The concentration and purity of the amplification products were tested by a Qubit fluorometer and agarose gel electrophoresis, and then, the amplification products were purified and pooled in equimolar quantities and sequenced by Novogene Bioinformatics Technology Company (Beijing, China) on an Illumina HiSeq platform using a 250 bp paired-end strategy (Caporaso *et al*., 2012). Raw sequencing data are available in the NCBI Sequence Read Archive database with the BioProject accession number PRJNA613376.

2.3. 16S sequences and statistical analyses After demultiplexing and trimming the barcodes and primers of each sample, the overlapping forward and reverse paired reads were assembled with Flash (Magoc and Salzberg, 2011). The assembled fastq files were processed using the open-source Quantitative Insights Into Microbial Ecology 2 (QIIME 2 (version 2018.11)) pipeline (https://qiime2.org; Hall and Beiko, 2018; Bolyen *et al*., 2019). We used default parameters in QIIME 2 (q2-quality-filter plugin) to conduct quality control, and then, the sequences were denoised by Deblur in QIIME 2 (q2-deblur-denoise plugin) (Amir *et al*., 2017). Sequences shorter than 250 bp were discarded (Bletz *et al*., 2017c). Subsequently, the chimeras were filtered using UCHIME (Edgar *et al*., 2011). Then, the rest of sequences were assigned into the sequence variants using the Greengenes database (version 13_8) (McDonald *et al*., 2012; DeSantis *et al*., 2006) through the q2-feature-classifier plugin, and the taxonomic composition at the phylum, family and genus levels was generated based on operational taxonomic units (OTU) annotation. After removing the sequences derived from chloroplasts, mitochondria, archaea and eukaryotes (q2-taxa-filter plugin), we removed the OTUs that covered < 0.005% of the total reads (q2-feature-table-filter plugin) (Bokulich *et al*., 2013). The number of reads per sample ranged from 21,420 to 154,799, and all samples were rarefied to 21,420 reads to mitigate the effects of uneven sampling (Schloss *et al*., 2013).

We used Faith's phylogenetic diversity (PD) (Faith, 1992) index to construct the rarefaction curve by randomly subsampling the data at a series of sequence depths to test whether the depth was sufficient to capture the majority of taxa (Supplementary Figure S1). Before using the rarefied dataset to estimate alpha and beta diversity of the skin bacteria by QIIME 2, a phylogenetic tree was created for generating phylogenetic diversity measures such as unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) or PD via the qiime alignment mafft, qiime alignment mask, qiime phylogeny fasttree and qiime phylogeny midpoint-root commands. Then, alpha and beta diversity metrics were assigned using the q2-diversity plugin. We estimated the observed OTUs, evenness, Shannon and PD of the skin bacteria to assess differences among samples. Kruskal–Wallis tests were used to compare alpha diversity among different species. Beta diversity was calculated with the Bray-Curtis, Jaccard, unweighted UniFrac (based on presence-absence and phylogenetic distance) and weighted UniFrac (based on abundance and phylogenetic distance) distance metrics and visualized with principal coordinates analysis (PCoA). We used permutational multivariate analyses of variance (PERMANOVA) (Anderson, 2005) with 999 permutations to determine the role of the host species in shaping skin bacterial communities. We performed further analyses and data visualization in the R environment, version 3.6.1 (R Core Team, 2019). We used the R package "ggplot2" (Wickham, 2016) to generate stacked bar charts at the phylum, family, and genus levels to visualize differences in the relative abundances of skin bacterial taxa. Using the same package, we visualized the differences in the alpha diversity of the five species. To examine differentially abundant bacterial taxa between the ectomorphs (habitat types) of samples, we used linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al*., 2011) on the Galaxy Web platform. We defined the dividing point as LDA ≥2.0 as recommended in the reference.

3. Results

We used 16S rRNA gene amplicon sequencing on the Illumina platform to examine skin bacterial communities from all 46 swab samples of each individual. After sequence filtration and classification, we detected a total of 1020 OTUs belonging to 17 phyla, with 9 phyla shared by all host species, 7 shared by 2–4 species and one found on only *M. fissipes*. Among the phyla, Proteobacteria (56–87%), Firmicutes (7–27%), Actinobacteria (3–11%) and Bacteroidetes (2–5%) were the dominant skin symbiont bacteria of all five host species (Figure 1). At the family level, Enterobacteriaceae was the most abundant in *M. fissipes*, *H. guentheri*, *F. multistriata*, and *R. zhenhaiensis* (21–50%), whereas Exiguobacteraceae was the most prevalent in *P. megacephalus* (22%) (Figure 1). At the genus level, *Escherichia* was the most abundant in *M. fissipes*, *H. guentheri*, and *F. multistriata*; an unnamed genus in Enterobacteriaceae was the most abundant in *R. zhenhaiensis* (18–21%); and *Exiguobacterium* was the most prevalent in *P. megacephalus* (22%) (Figure 2).

We observed significant differences in the alpha diversity of the skin bacteria among host species (Shannon, Kruskal–Wallis: $H = 19.1905$, $P = 0.0007$; PD, Kruskal–Wallis: $H = 17.5626$, $P = 0.0015$; observed OTUs, Kruskal–Wallis: $H = 19.2590$, $P = 0.0007$; evenness, Kruskal–Wallis: $H = 15.2537$, $P = 0.0042$). Multiple

Table 1 Sampling locations for five amphibian species from Lishui, Zhejiang, China.

Species	Family	*N*	Coordinates
Fejervarya multistriata	Dicroglossidae	11	N28.463601° E119.936487°
Hylarana guentheri	Ranidae	11	N28.461126° E119.900026°
Microhyla fissipes	Microhylidae	9	N28.460355° E119.938849°
Polypedates megacephalus	Rhacophoridae	5	N28.439375° E119.913218°
Rana zhenhaiensis	Ranidae	10	N28.463867° E119.936191°

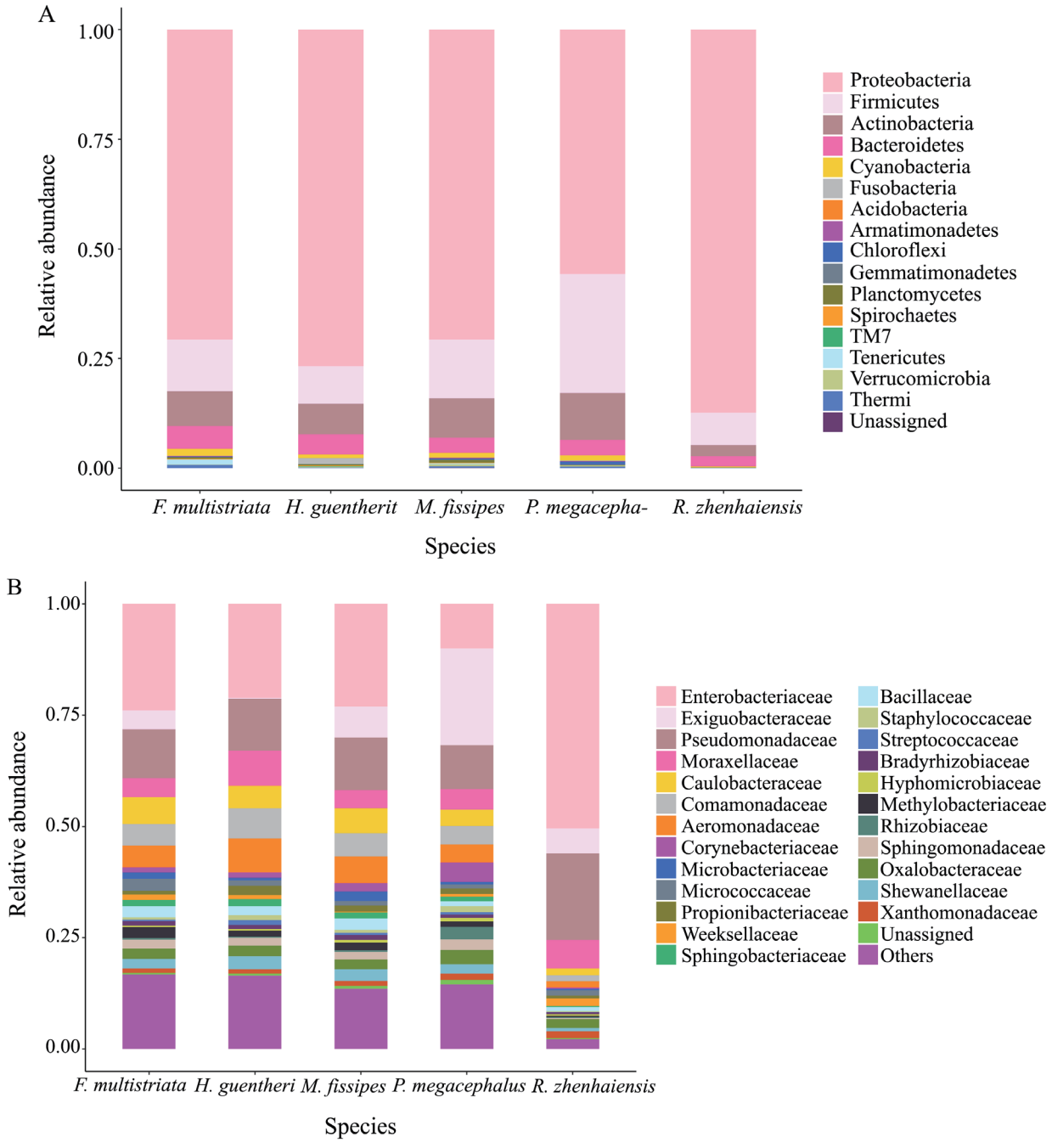

Figure 1 Relative abundance of skin bacterial taxa of five host species. A) Relative abundance of skin bacterial taxa at the phylum level across species. B) Relative abundance of skin bacterial taxa at the family level across species. Taxa with relative abundances < 1% were clustered together.

comparisons showed that the Shannon index and PD were significantly lower in *R. zhenhaiensis* than in other species (Table 2, Figure 3), and the results of the observed OTUs and evenness were similar to the Shannon index and PD results (Table S1).

For beta diversity of the skin bacteria, PERMANOVA analyses showed that the host species indeed had a significant effect on both the bacterial community composition (Jaccard, *Pseudo-F* = 1.4747, *P* = 0.001; unweighted UniFrac, *Pseudo-F* = 1.9046, *P* = 0.001) and abundance pattern (Bray-Curtis distance, *Pseudo-F* = 2.3398, *P* = 0.002; weighted UniFrac, *Pseudo-F* = 3.2569, *P* = 0.001). Pairwise comparisons by PERMANOVA indicated that there were differences in bacterial composition among most species pairs, especially between *R. zhenhaiensis* and each of the other four species (Table 3, Table S2).

Given the unique alpha and beta diversity of the skin bacteria of *R. zhenhaiensis*, the only species sampled from land, we further explored the role of habitat type or sampling site type on the skin symbiont of the host using LEfSe to identify specific taxa that varied in abundance in the samples sourced from aquatic and terrestrial environments. Interestingly, Actinobacteria, Bacteroidetes, Cyanobacteria and Firmicutes were highly abundant in *F. multistriata*, *H. guentheri*, *M. fissipes* and *P. megacephalus* samples from watersides areas; however, *R. zhenhaiensis* sampled from land contained more Proteobacteria than other species (Figure 5).

4. Discussion

In this study, we used skin swabs of five co-habiting species to explore the diversity of the skin bacterial communities by using 16S rRNA amplicon sequencing and to describe and assess the traits, interspecies differences and effects of ecomorphology on skin symbionts of five amphibian species in southeastern China. We found Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes dominated all five host species, but the alpha and beta diversity and the main taxa at the family and genus level of the skin bacteria were different among host species. Lower alpha diversity and richer Proteobacteria were found on the terrestrial species *R. zhenhaiensis* than on the four aquatic species, *F. multistriata*, *H. guentheri*, *M. fissipes* and *P. megacephalus*.

This is among the first studies to seek the skin symbionts of multiple species and analyse them together in southeastern

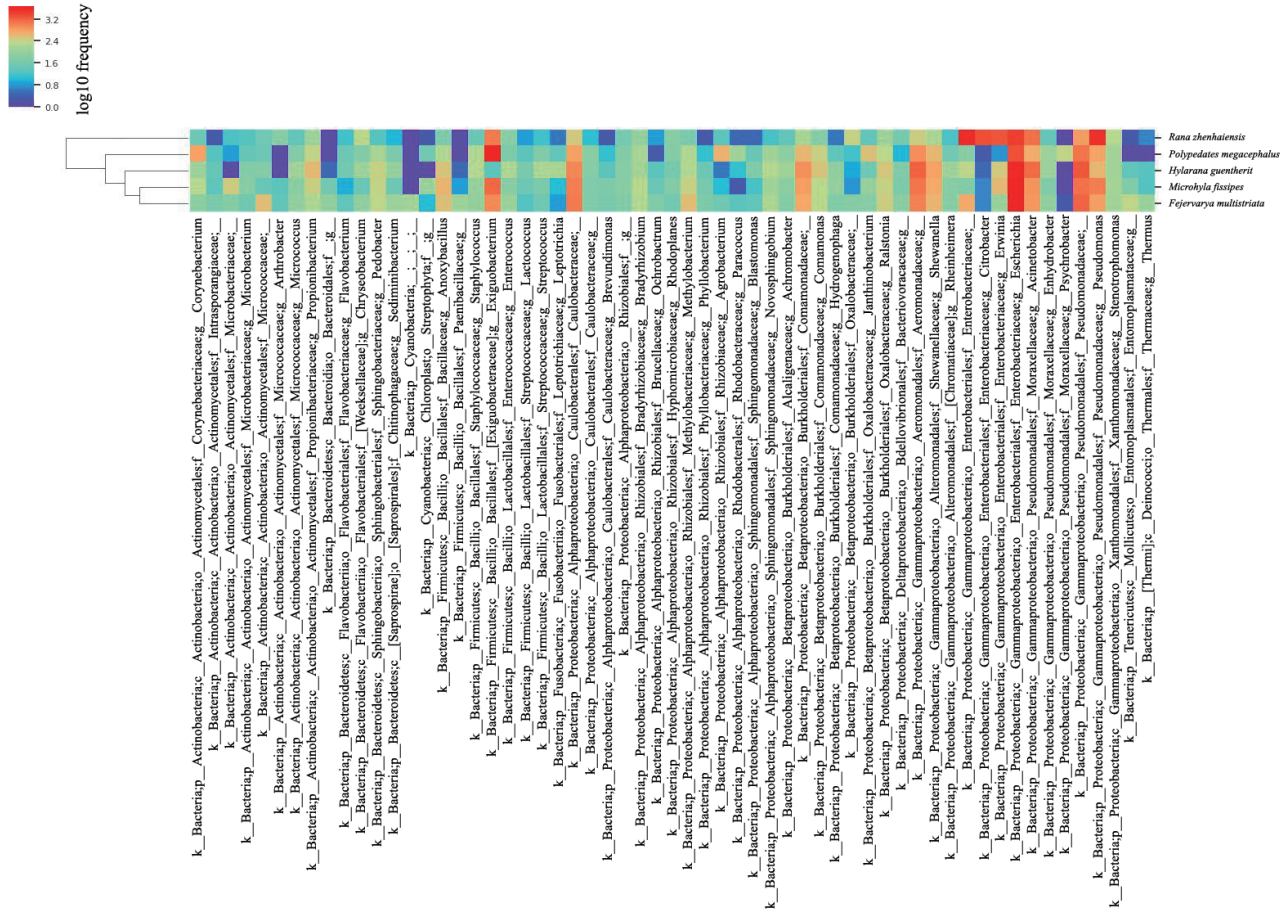

Figure 2 Heatmap of bacterial genera with relative abundances > 0.1% across five host species based on Bray-Curtis distance.

Table 2 Multiple comparisons of species pairs by Kruskal–Wallis tests for Shannon index and PD determination among host species. Boldface indicates significance < 0.05.

Index	Group 1	Group 2	H	*p*-value	*q*-value
Shannon	*F. multistriata* (*n*=11)	*H. guentheri* (*n*=11)	1.1739	0.2786	0.4643
		M. fissipes (*n*=9)	0.1169	0.7324	0.8633
		P. megacephalus (*n*=5)	0.0802	0.777	0.8633
		R. zhenhaiensis (*n*=10)	10.4926	0.0012	**0.0048**
	H. guentheri (*n*=11)	*M. fissipes* (*n*=9)	1.3867	0.239	0.4643
		P. megacephalus (*n*=5)	0.0032	0.9548	0.9548
		R. zhenhaiensis (*n*=10)	14.4595	0.0001	**0.0014**
	M. fissipes (*n*=9)	*P. megacephalus* (*n*=5)	0.36	0.5485	0.7836
		R. zhenhaiensis (*n*=10)	10.14	0.0015	**0.0048**
	P. megacephalus (*n*=5)	*R. zhenhaiensis* (*n*=10)	6	0.0143	**0.0358**
PD	*F. multistriata* (*n*=11)	*H. guentheri* (*n*=11)	0.0528	0.8182	0.9548
		M. fissipes (*n*=9)	0.6364	0.425	0.7084
		P. megacephalus (*n*=5)	0.0032	0.9548	0.9548
		R. zhenhaiensis (*n*=10)	10.4926	0.0012	**0.006**
	H. guentheri (*n*=11)	*M. fissipes* (*n*=9)	0.7633	0.3823	0.7084
		P. megacephalus (*n*=5)	0.0032	0.9548	0.9548
		R. zhenhaiensis (*n*=10)	12.3967	0.0004	**0.0043**
	M. fissipes (*n*=9)	*P. megacephalus* (*n*=5)	0.36	0.5485	0.7836
		R. zhenhaiensis (*n*=10)	8.1667	0.0043	**0.0121**
	P. megacephalus (*n*=5)	*R. zhenhaiensis* (*n*=10)	7.935	0.0048	**0.0121**

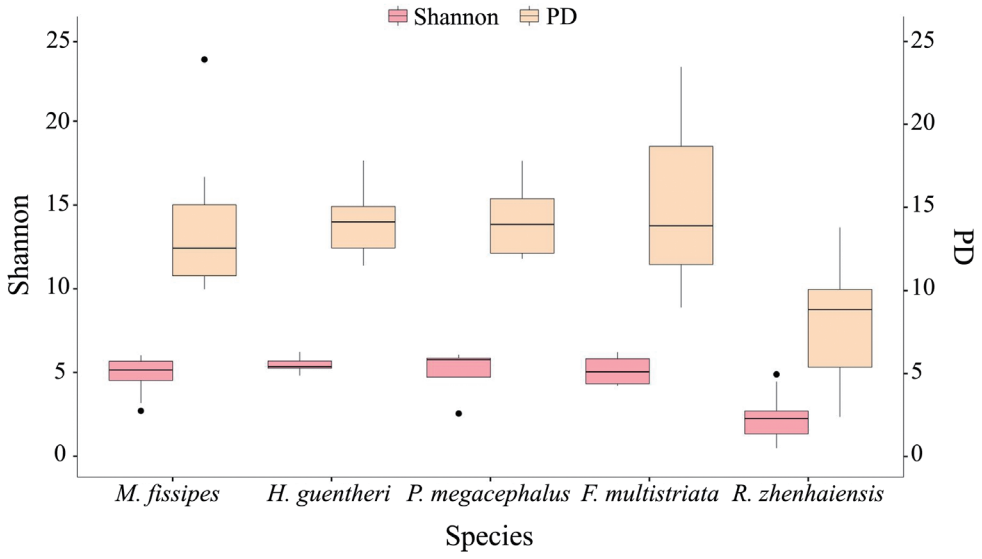

Figure 3 Shannon index and PD of skin bacteria among the five host species.

China, where diverse amphibians are distributed. A detailed understanding of amphibian skin symbionts in different regions of the globe could aid the exploration of measures to mitigate chytridiomycosis (Abarca *et al.*, 2018). Southeastern China has been proven to be a suitable habitat for *Bd*, as inferred by models (Liu *et al.*, 2013); however, large-scale population declines and extinctions have not been detected by extensive field surveys (Bai *et al.*, 2012; Zhu *et al.*, 2014). One of the possible reasons for this is that *Bd* originated in Asia and has a long coevolutionary history with amphibians in Asia (O'Hanlon *et al.*, 2018). Another important factor is the beneficial effect of the skin microbiome on the amphibian host through microbial species diversity, community composition and assemblage of anti-*Bd* bacterial taxa, which can produce anti-fungal compounds (Harris *et al.*, 2006; Loudon *et al.*, 2014; Jani *et al.*, 2017; Bell *et al.*, 2018). Considering the close relationship between the skin symbionts and the host, it is of great significance to explore the skin symbionts of amphibians

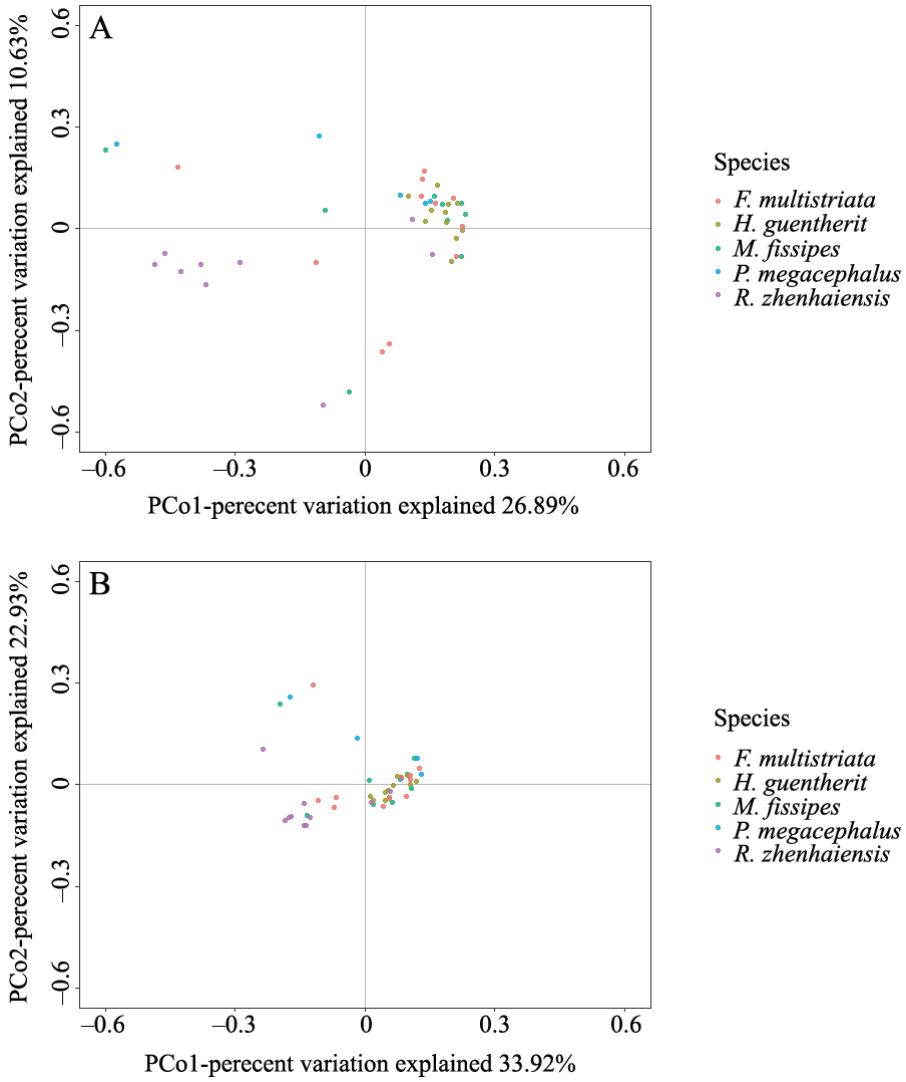

Figure 4 Principal coordinate analysis (PCoA) plots of the beta diversity of the skin bacterial community. A) Bray-Curtis matrices and B) weighted UniFrac matrices of five host species. Each point represents the skin bacterial community of an individual sample.

in Asia to understand host health and disease resistance in this region.

It has been reported in various studies that host ecology or habitat and/or host taxonomy are crucial drivers of skin microbial composition and diversity in amphibians (McKenzie *et al.*, 2012; Bletz *et al.*, 2017b). We found clear differences in the skin bacteria of the five frog host species that we assessed and pronounced differences in ecomorphology between riparian and terrestrial species in terms of both of the skin microbial diversity and composition. With regard to the Shannon index and PD, the main difference was reflected in ecomorphology; however, contrary to previous studies that showed that terrestrial amphibians have greater bacterial diversity than arboreal or aquatic species (Abarca *et al.*, 2018; Rebollar *et al.*, 2019), we found that the bacterial diversity of *R. zhenhaiensis* was significantly lower than that of other riparian frogs. Nevertheless, the Shannon index of the skin bacteria of *Craugastor fitzingeri*, a terrestrial toad in Panama, exhibited a median value among the riparian, arboreal and terrestrial amphibians (Rebollar *et al.*, 2016). One of the possible explanations for the results is that the environmental conditions, such as temperature, humidity and altitude, varied in the sampling areas. Alternatively, while our study and Rebollar *et al.* (2016) contained only one terrestrial species, the studies by Abarca *et al.* (2018) and Rebollar *et al.* (2019) lacked aquatic species. Therefore, studies on additional host species inhabiting different environments around the world are needed to test whether the difference in alpha diversity among ecomorphologies is ubiquitous or limited to specific species. Furthermore, unlike the results for the gut microbiota,

Table 3 Multiple comparisons by PERMANOVA of β-diversity among species based on Bray-Curtis and weighted UniFrac distance matrixes. Boldface indicates significance < 0.05.

index	Group 1	Group 2	Sample size	pseudo-*F*	*p*-value	*q*-value
Bray-Curtis	*F. multistriata*	*H. guentheri*	22	1.5416	0.042	0.07
		M. fissipes	20	0.4619	0.978	0.978
		P. megacephalus	16	1.3552	0.15	0.1875
		R. zhenhaiensis	21	3.2958	0.001	**0.0033**
	H. guentheri	*M. fissipes*	20	1.2595	0.141	0.1875
		P. megacephalus	16	2.8489	0.001	**0.0033**
		R. zhenhaiensis	21	5.5809	0.001	**0.0033**
	M. fissipes	*P. megacephalus*	14	1.0217	0.409	0.4544
		R. zhenhaiensis	19	2.5287	0.017	**0.0425**
	P. megacephalus	*R. zhenhaiensis*	15	1.7858	0.04	0.07
weighted UniFrac	*F. multistriata*	*H. guentheri*	22	1.5872	0.105	0.15
		M. fissipes	20	0.3882	0.909	0.909
		P. megacephalus	16	1.1978	0.273	0.3413
		R. zhenhaiensis	21	5.3213	0.001	**0.005**
	H. guentheri	*M. fissipes*	20	1.6926	0.034	0.0567
		P. megacephalus	16	2.8789	0.006	**0.012**
		R. zhenhaiensis	21	11.4543	0.001	**0.005**
	M. fissipes	*P. megacephalus*	14	0.7413	0.572	0.6356
		R. zhenhaiensis	19	4.3736	0.005	**0.012**
	P. megacephalus	*R. zhenhaiensis*	15	5.3313	0.002	**0.0067**

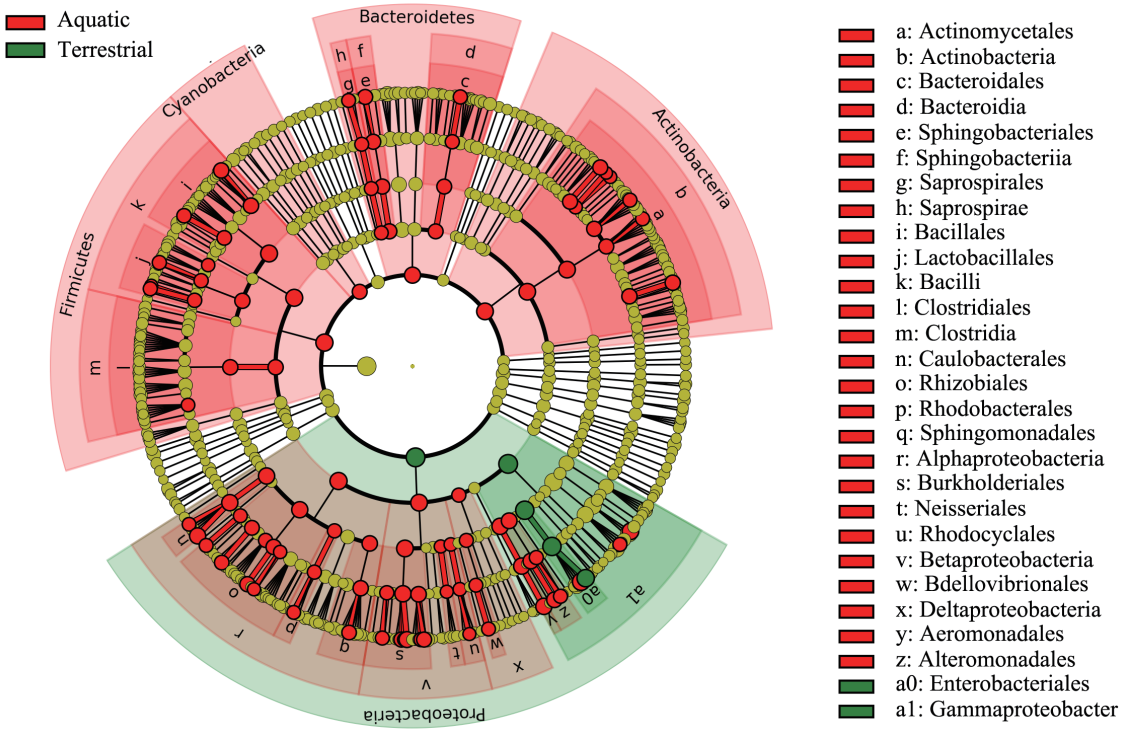

Figure 5 Cladograms showing that the bacterial taxa differ between species sampled from aquatic and terrestrial environments based on LEfSe.

we found no evidence for strong phylosymbiosis in these skin microbial communities of the five frog hosts, that is, we did not find skin microbial communities parallel with host phylogeny (Brooks *et al*., 2016). This is consistent with the result of Bletz *et al*., 2017b. In addition to the difference in coevolution between gut and skin with the bacterial symbionts elucidated

by Bletz, it is plausible that skin bacteria varied constantly via environmental transmission (Loudon *et al*., 2014), and probable physical contact between species living in the same or adjacent habitats caused this effect.

In the present study, we found 17 bacterial phyla on the amphibian skin, among which Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the four dominant phyla, which is consistent with observations in previous studies conducted in both temperate and tropical systems (e.g., Walke *et al*., 2014; Belden *et al*., 2015; Bletz *et al*., 2017b; Jiménez *et al*., 2019), suggesting that the skin of amphibians may be a selective niche for the colonization of peculiar bacterial taxa (Rebollar *et al*., 2016; Bletz *et al*., 2017b). However, the relative abundances of these phyla seemed to vary to some extent across different areas; Proteobacteria was almost the most abundant phylum on amphibians around the world, and the other main phyla may be shifted (Belden *et al*., 2015; Bletz *et al*., 2017b; Abarca *et al*., 2018). In this study, Proteobacteria was more abundant on the terrestrial species *R. zhenhaiensis* than on the other species, which is similar to the results of other studies (Belden *et al*., 2015; Abarca *et al*., 2018). Proteobacteria was also the most abundant of summer and wild samples of skin microbiotas on the terrestrial species *R. dybowskii* in northeastern China (Bie *et al*., 2019; Tong *et al*., 2019). In southeastern China, Firmicutes was the second most abundant phylum detected on the skin of amphibians, which was different from the results obtained in some tropical countries, such as Panama, Costa Rica and Madagascar (Belden *et al*., 2015; Bletz *et al*., 2017b; Jiménez *et al*, 2019). Apparently, the frogs of temperate regions may have higher Firmicutes abundance than those of tropical zones (Abarca *et al*., 2018).

At the family and genus levels, Enterobacteriaceae and Exiguobacteraceae and the genera *Escherichia* and *Exiguobacterium* belonging to these two families were identified as the most abundant taxa on the skin of our focal species. This result is interesting in light of largely similar studies that showed that the most dominant taxon was Pseudomonadaceae or *Pseudomonas* in different areas (Becker *et al*., 2015; Rebollar *et al*., 2016, 2018; Prado-Irwin *et al*., 2017; Catenazzi *et al*., 2018; Bie *et al*., 2019). However, Enterobacteriaceae was the most abundant family on *Mantella aurantiaca* in Madagascar (Passos *et al*., 2018). Interestingly, Enterobacteriaceae and Pseudomonadaceae often exhibit high relative abundances together on the skin of many amphibians distributed in many countries, such as Madagascar, Germany, USA, and Australia (Bletz *et al*., 2017b, c, d; Kearns *et al*., 2017; Bell *et al*., 2018). Additionally, a high proportion of inhibitory isolates belonging to Enterobacteriaceae and Pseudomonadaceae was found in the database of culturable anti-*Bd* bacteria (Woodhams *et al*., 2015). With regard to the other abundant genus, *Exiguobacterium*, in our study, also showed high relative abundance in a population of common midwife toads (*Alytes obstetricans*) (Bates *et al*., 2018). To date, a total of 17 species have been found to belong to *Exiguobacterium*, and the isolates of this genus have been found to exhibit bioremediation and enzyme production and degradation of toxic substances. *Exiguobacterium* is a versatile bacterial genus distributed in a variety of environments (Kasana and Pandey, 2018). The high abundance of Enterobacteriaceae and Exiguobacteraceae in this study may indicate the putatively unique anti-*Bd* function among the five sympatric frog species in southeastern China. Of course, further study is needed to verify this hypothesis through bacterial isolation and culture techniques.

To our knowledge, this is the first study to assess differences in the skin microbiota of cooccurring species in southeastern China by high-throughput sequencing. It is important to describe and understand indigenous skin bacterial communities to develop conservation strategies that can be applied locally. Further sampling across a wide area of China is needed to promote the understanding of amphibian skin microbial ecology and to obtain an improved understanding of the particular mechanism of interaction between *Bd* and host amphibians in China.

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Appendix

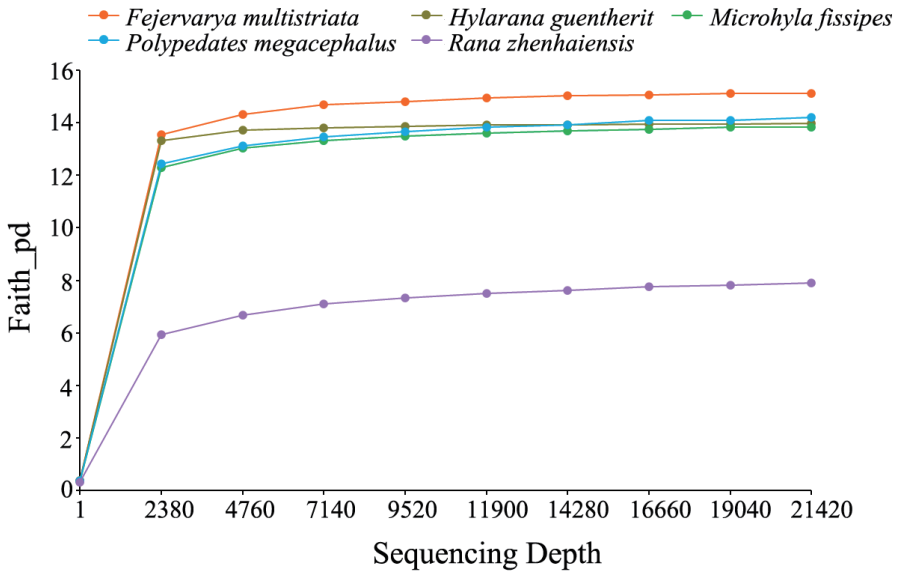

Figure S1 Rarefaction curves based on the phylogenetic diversity measure of skin bacterial samples from five host species.

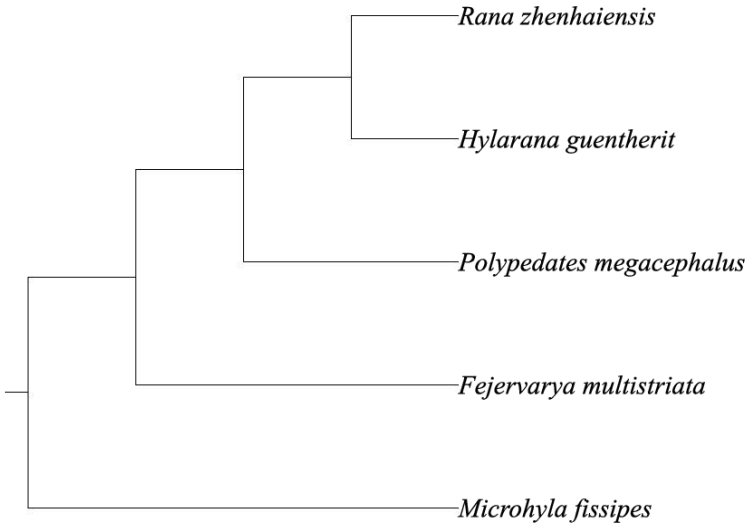

Figure S2 Phylogenetic tree constructed by Phylogenetic Generalized Least Squares (PGLS) processed by R based on the amphibian tree constructed by Peloso *et al.*, 2015. We have not found the species Microhyla fissipes in Peloso *et al.*, 2015, therefore we used the closely related species Microhyla heymonsi to instead of Microhyla fissipes (Matsui *et al.*, 2011).

Table S1 Multiple comparisons of species pairs by Kruskal–Wallis tests for observed OTUs and evenness among host species. Boldface indicates significance < 0.05.

Index	Group 1	Group 2	H	*p*-value	*q*-value
OTUs	*F. multistriata* (*n*=11)	*H. guentheri* (*n*=11)	0.6737	0.4118	0.5882
		M. fissipes (*n*=9)	0.417	0.5184	0.632
		P. megacephalus (*n*=5)	0.1157	0.7338	0.7338
		R. zhenhaiensis (*n*=10)	9.3953	0.0022	**0.0054**
	H. guentheri (*n*=11)	*M. fissipes* (*n*=9)	0.3247	0.5688	0.632
		P. megacephalus (*n*=5)	3.4941	0.0616	0.1232
		R. zhenhaiensis (*n*=10)	12.4047	0.0004	**0.0043**
	M. fissipes (*n*=9)	*P. megacephalus* (*n*=5)	1.2844	0.2571	0.4285
		R. zhenhaiensis (*n*=10)	10.4199	0.0012	**0.0054**
	P. megacephalus (*n*=5)	*R. zhenhaiensis* (*n*=10)	9.3918	0.0022	**0.0054**
Evenness	*F. multistriata* (*n*=11)	*H. guentheri* (*n*=11)	0.3115	0.5767	0.8647
		M. fissipes (*n*=9)	0.2439	0.6214	0.8647
		P. megacephalus (*n*=5)	0.1572	0.6917	0.8647
		R. zhenhaiensis (*n*=10)	9.6	0.0019	**0.0097**
	H. guentheri (*n*=11)	*M. fissipes* (*n*=9)	0.5209	0.4704	0.8647
		P. megacephalus (*n*=5)	0.0032	0.9548	0.9548
		R. zhenhaiensis (*n*=10)	11.9058	0.0006	**0.0056**
	M. fissipes (*n*=9)	*P. megacephalus* (*n*=5)	0.04	0.8415	0.935
		R. zhenhaiensis (*n*=10)	7.26	0.0071	**0.0235**
	P. megacephalus (*n*=5)	*R. zhenhaiensis* (*n*=10)	4.86	0.0275	0.0687

Table S2 Multiple comparisons by PERMANOVA of β-diversity among species based on Jaccard and unweighted UniFrac distance matrixes. Boldface indicates significance < 0.05.

index	Group 1	Group 2	Sample size	pseudo-*F*	*p*-value	*q*-value
Jaccard	*F. multistriata*	*H. guentheri*	22	1.5271	0.007	**0.014**
		M. fissipes	20	1.0016	0.403	0.403
		P. megacephalus	16	1.3742	0.037	0.0422
		R. zhenhaiensis	21	1.791	0.002	**0.01**
	H. guentheri	*M. fissipes*	20	1.1482	0.038	**0.0422**
		P. megacephalus	16	1.3975	0.004	**0.01**
		R. zhenhaiensis	21	2.0176	0.001	**0.01**
	M. fissipes	*P. megacephalus*	14	1.224	0.03	**0.0422**
		R. zhenhaiensis	19	1.5312	0.004	**0.01**
	P. megacephalus	*R. zhenhaiensis*	15	1.4738	0.021	**0.035**
Unweighted UniFrac	*F. multistriata*	*H. guentheri*	22	1.664	0.036	0.072
		M. fissipes	20	0.9737	0.397	0.397
		P. megacephalus	16	1.6258	0.061	0.1017
		R. zhenhaiensis	21	3.0558	0.002	**0.01**
	H. guentheri	*M. fissipes*	20	1.0328	0.392	0.397
		P. megacephalus	16	1.1606	0.178	0.2543
		R. zhenhaiensis	21	3.2781	0.002	**0.01**
	M. fissipes	*P. megacephalus*	14	1.045	0.375	0.397
		R. zhenhaiensis	19	2.0336	0.018	**0.045**
	P. megacephalus	*R. zhenhaiensis*	15	2.3715	0.017	**0.045**